

## Endocytosis in alveolar type II cells: Effect of charge and size of tracers

(cationic ferritin/multivesicular bodies/lamellar bodies/transepithelial transport)

MARY C. WILLIAMS

Cardiovascular Research Institute and Department of Anatomy, University of California, San Francisco, CA 94143

Communicated by John A. Clements, June 22, 1984

**ABSTRACT** The type II cell of the alveolar epithelium of adult rats has been studied by electron microscopy to determine its ability to endocytose electron-dense tracers of differing molecular charge and size. The tracers studied were native (anionic) ferritin, cationic ferritin, 70-kilodalton dextran, and colloidal carbon. Alveolar macrophages ingested all tracers in large amounts, while type II cells took up significant amounts of cationic ferritin only. This tracer was observed, in sequence, within small pinocytic vesicles, large electron-lucent multivesicular bodies, small electron-dense multivesicular bodies, and, by 30 min after instillation, within the nonlamellar matrix of lamellar bodies. By 2 hr all lamellar bodies in any labeled cell contained cationic ferritin. Cationic ferritin also appeared to be transported from alveoli to interstitium by vesicles of type II, but not type I, cells. Native ferritin and dextran were observed in the same organelles as cationic ferritin but in much smaller amounts; colloidal carbon was not taken up by type II cells. These tracers were not observed in the alveolar interstitium. Type II cells therefore appear to internalize preferentially the tracer that binds to the cell membrane. Once within the cell, the tracer may enter a pathway that terminates in a lamellar body or, in the case of cationic ferritin, may be ferried across the cell.

Results of many studies suggest that the ability to ingest materials by endocytosis is a property shared by most, if not all, cells. The general characteristics of the several types of endocytosis have been reviewed recently by several groups (1-3). It has been assumed that this function is also performed by the type II cell of the alveolar epithelium. Occasional early observations suggested that this was indeed the case (4, 5). More recent observations which indicate that type II cells recycle pulmonary surfactant, a material that they synthesize, have prompted renewed interest in endocytic mechanisms in type II cells and in the pathways taken by materials ingested by these cells. On the basis of the observed rates of clearance from alveoli of several surfactant-associated phospholipids, Hallman *et al.* (6) proposed that recycling of surfactant may take place by means of a process of bulk uptake. If this were true, it might be expected that a vesicle-mediated pathway would link alveoli with lamellar bodies, the intracellular storage granules of surfactant.

This study was therefore undertaken to define the fate of various tracer molecules placed in small quantities in alveoli of intact animals in order to determine how the molecular charge and size of the tracers influenced the uptake, if any, by alveolar epithelial cells and, second, to characterize the intracellular pathways taken by the tracers after uptake. Studies of a similar nature have been carried out to determine the fate of several lectins, one of which binds specifi-

cally to the apical plasma membrane of type II cells. These results will be reported later.

The observations of these two groups of studies suggest that, like other cells, type II cells internalize most rapidly and in greatest amounts those tracers that bind to the apical plasma membrane; binding may be the result of either molecular charge or specific interaction with a membrane glycoprotein. The internalized tracers are cycled from vesicles to multivesicular bodies and thence to lamellar bodies or, depending on the tracer used, to the basal cell surface from which they are expelled into the underlying interstitium. Certain of these results have been reported previously in an abstract (7).

### METHODS

**Animals and Administration of Tracers.** All studies were carried out on 275- to 350-g male Long-Evans rats. For instillation of tracers the animals were anesthetized with sodium methohexital at a dosage of 7 mg/100 g of body weight. They were then intubated intratracheally by the insertion of a 16-gauge Teflon catheter via the oral cavity. A length of polyethylene tubing (PE-10, 0.61 mm outside diameter) was threaded through the catheter and pushed gently into the lung until it met a slight resistance. Solutions of the tracers (see below) were rapidly instilled into the lung through the small tubing. Both catheters were immediately removed. Cannulation and instillation of tracers were accomplished in 30-60 sec. At intervals ranging from 10 min to 18 hr the animals were reanesthetized and the lungs were fixed by vascular perfusion or intratracheal instillation as reported previously (8).

**Fixations.** Lungs of ferritin-labeled animals were fixed in 2% (wt/vol) glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium phosphate buffer, at 37°C and pH 7.4. They were postfixed in osmium tetroxide, block stained in uranyl acetate, dehydrated in cold acetone solutions, and embedded in Epon as described previously (9). Thin sections were studied in a Zeiss EM-10 electron microscope without further staining of the grids. Tissues from dextran-labeled animals were fixed by the method of Simionescu *et al.* (10).

**Tracers.** Native (anionic, pI 4.6) ferritin (NF) was purchased from Miles and was dialyzed against Gey's balanced salt solution (GBSS) buffered with 10 mM Hepes, pH 7.4, prior to use. Cationic ferritin (CF, pI 8.5), suspended in isotonic saline, was also purchased from Miles. Both tracers (600 kilodaltons,  $\approx 10$  nm diameter) were used at protein concentrations of 1 mg/ml. Dextran (T-70, 70 kilodaltons,  $\approx 12$  nm diameter), purchased from Pharmacia (Uppsala, Sweden), was dissolved in GBSS at a concentration of 200 mg/ml. Biological grade colloidal carbon, obtained from Gunther-Wagner (Hanover, F.R.G.), was dialyzed against GBSS before use. The diameter of the carbon particles is about 25 nm. Tracers were administered in volumes of

100–150  $\mu$ l per animal, to which was added 20  $\mu$ l of colloidal carbon to determine at the time of fixation the area into which the tracers had been deposited; this was generally the lower right lobe. Tissue blocks were selected for electron microscopy only if carbon was observed in 0.5- $\mu$ m-thick sections by light microscopy.

The numbers of animals studied for each tracer and the range of times at which tissues were collected were as follows: NF, 5 animals, 30 min to 18 hr; CF, 16 animals, 10 min to 18 hr; dextran, 5 animals, 60 min to 6 hr; and colloidal carbon, 26 animals, 10 min to 18 hr.

## RESULTS

Careful examination of cellular ultrastructure of areas of lung that contained the various tracers revealed little evidence of tissue or cellular damage resulting from intubation or instillation of tracers. At early times (10–30 min), small distended areas, suggestive of the presence of edema fluid, were occasionally observed in the alveolar interstitium. Epithelial tight junctions were morphologically intact, however, and none of the tracers was seen within the length of the tight junctions or in the subjacent intercellular space. Observations on one animal injected with dextran were discarded because of the presence of numerous neutrophils in both the interstitium and alveoli; this was not observed in other animals.

As anticipated, alveolar macrophages internalized all tracers, including carbon, at all time points and in all animals studied. The tracers were present in large amounts in intracellular vesicles of various sizes and shapes. Because the primary interest in this study was to follow uptake of tracers by cells of the alveolar epithelium, labeling of macrophages was used only to verify that both tracers were present in the area of lung selected for study and that the tracers could be readily visualized. Alveolar macrophage uptake thus served as a positive control with which to compare endocytic activity of types I and II epithelial cells.

CF. The tracer internalized most rapidly and in greatest amounts by type II cells was CF, which adhered in small clusters to the apical plasma membrane (Fig. 1). After 10 min, the earliest time examined, CF was present in small vesicles in the upper third of the cell (Fig. 1). Most of the CF-

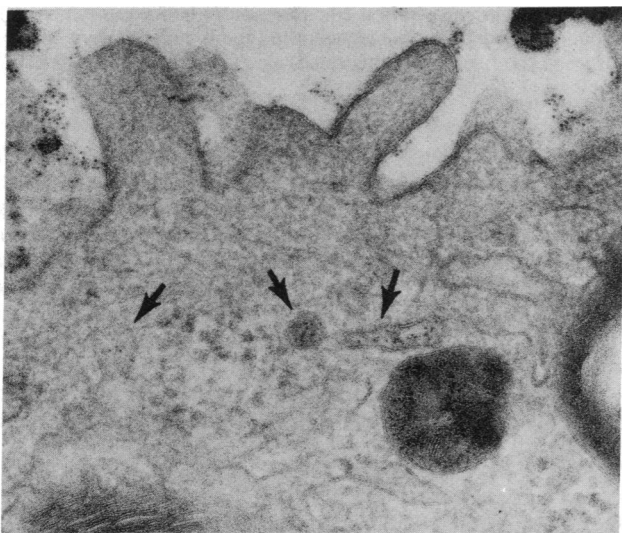


FIG. 1. After its instillation into the lung, CF adheres in small clusters to the type II cell plasma membrane and is present within numerous small vesicles in the apical cytoplasm (arrows) as well as a small lamellar body. These compartments are illustrated in this section taken from an animal exposed to CF for 60 min. ( $\times 67,500$ .)

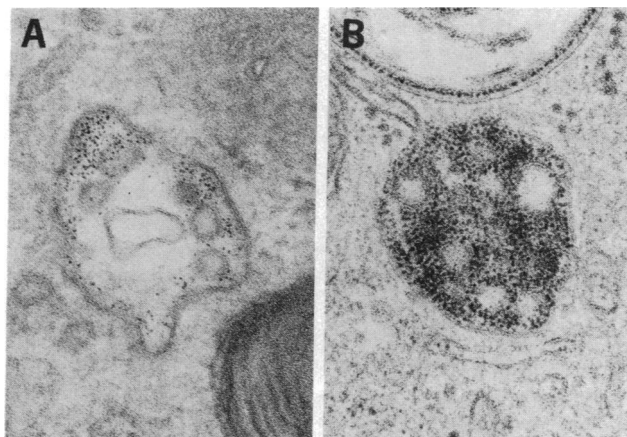


FIG. 2. (A) After uptake by vesicles, CF next appears in irregularly shaped electron-lucent MVBs, which often contain a larger internal vesicle as seen here in a 10-min specimen. ( $\times 72,000$ .) (B) A second population of MVBs, labeled somewhat later as in this 30-min specimen, consists of smaller, electron-dense structures, which generally contain lysosomal enzymes (11). This population of MVBs appears to fuse with LBs (12). ( $\times 72,000$ .)

containing vesicles had a diameter less than 95 nm, although a few were as large as 260 nm (range of 30 vesicles, 40–260 nm). Some vesicles were clathrin coated. A few electron-lucent multivesicular bodies (MVBs) also contained CF (Fig. 2A), but the tracer was not present in the smaller, electron-dense MVBs at this time. The MVBs labeled first frequently contained a single large irregularly shaped vesicle in addition to the more typical small vesicles (Fig. 2A). Images suggestive of fusion of CF-laden vesicles with MVBs were common. By 30 min, both types of MVBs were labeled with CF in the intervesicular space (Fig. 2). No tracers were observed within the lumens of the small internal vesicles. At this time, a few lamellar bodies (LBs) also contained CF within their nonlamellar material; cytochemistry indicates that this substance contains certain lysosomal enzymes (11). LBs, MVBs, and vesicles continued to accumulate CF with longer exposure (Figs. 3 and 4). By about 60–120 min, all LBs in any one labeled cell contained CF. There did not appear to be preferential labeling of any subpopulation of LBs, whether related to size or to position within the cell. At later times, and perhaps related to an increase in the concentration of CF in LBs, the tracer became intercalated between individual

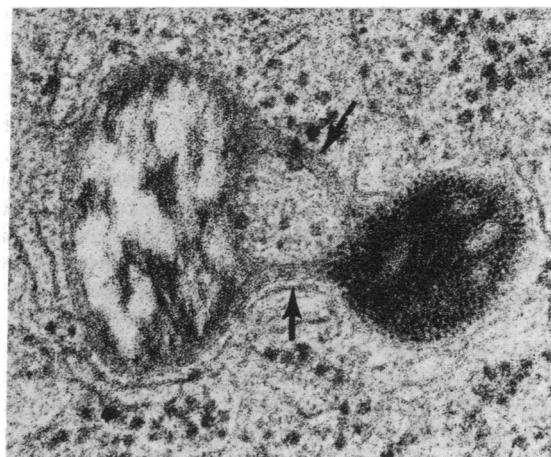


FIG. 3. A CF-laden MVB is connected to a small LB by long extensions; this connection presumably facilitates entry of MVB contents into LBs. ( $\times 80,000$ .)

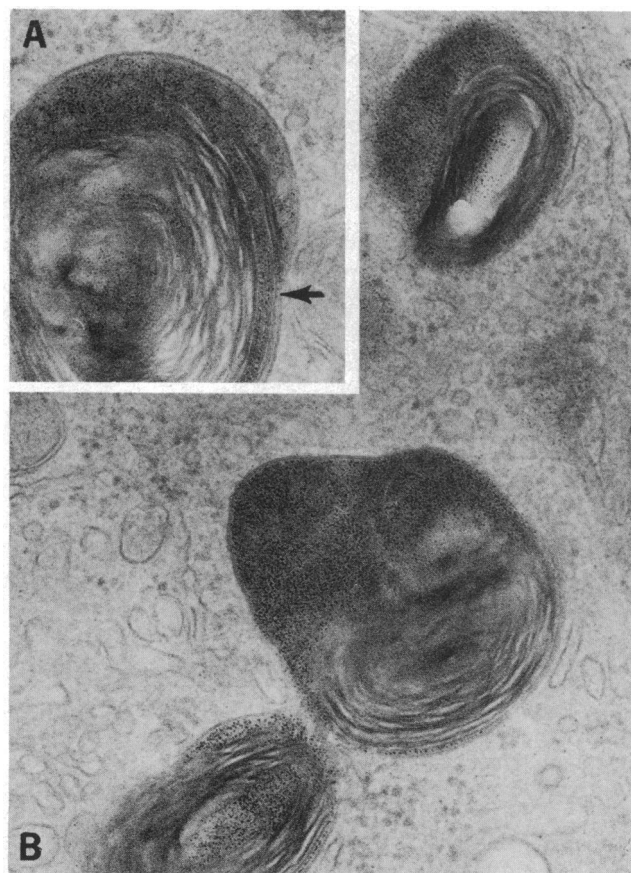


FIG. 4. As illustrated in this 7-hr specimen, with time all LBs of any labeled cell contain CF (A), which may eventually intercalate between phospholipid lamellae (B). (A,  $\times 54,000$ ; B,  $\times 52,000$ .)

phospholipid lamellae (Fig. 4B). Although infrequent, a few CF-labeled vesicles were observed at the periphery of the Golgi complex; in no instance did the tracer enter compartments clearly identified as Golgi constituents. Starting at about 2 hr, and accumulating with time, small aggregates of 5–15 molecules of CF were present in the interstitial compartment between the basal plasma membrane of the type II cell and the lamina densa of the epithelial basement membrane (Fig. 5A). The clusters of CF were distributed with a periodicity similar to that observed for ruthenium red-stained anionic sites in the alveolar basement membrane (13). Small CF-containing vesicles were present in the basal cytoplasm, but none was observed discharging its content of tracer. By 18 hr after instillation, no CF was adherent to the type II cell plasma membrane, and no CF-labeled pinocytic vesicles were observed. CF was still present in LBs at this time, but most MVBs were unlabeled. Very little CF was internalized by type I cells, although the extensive surfaces of these cells were often covered with the tracer and the cells contained abundant pinocytic vesicles (Fig. 5B). In two instances only, CF was observed in small vesicles in the perinuclear and thin cellular region of type I cells, respectively. No CF was observed in the interstitium underlying type I cells (Fig. 5B).

**NF.** Neither cell type internalized NF to any extent, although it was present in adjacent alveoli, and NF-laden alveolar macrophages were nearby. The amount of NF in the interstitial space was also negligible (Fig. 5C). Scattered molecules were found in vesicles, MVBs, and LBs of type II cells, but the total amount was always quite small and most of these organelles contained no tracer (Fig. 6A).

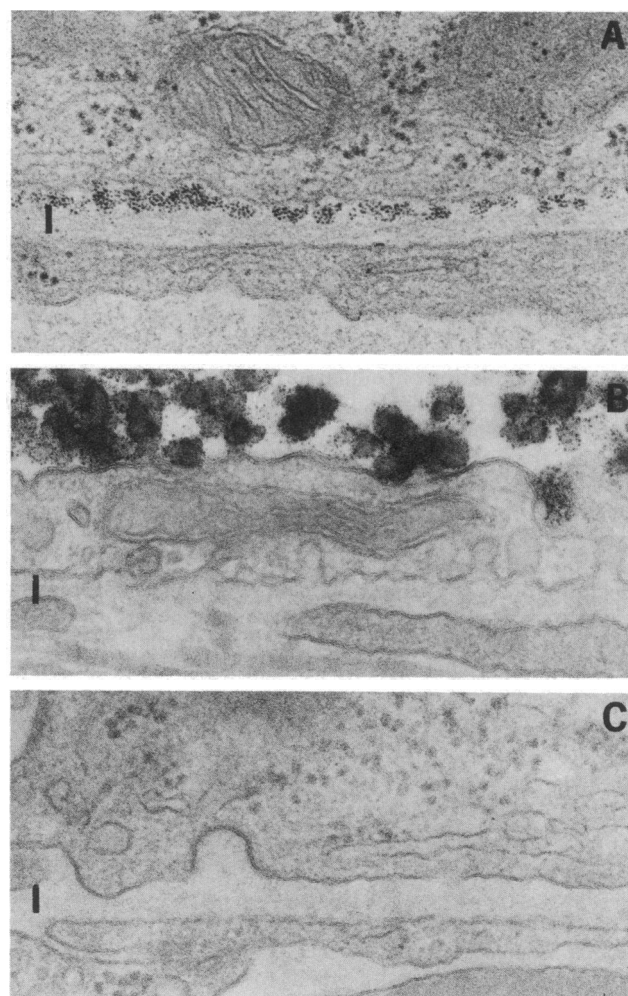


FIG. 5. (A) Beginning at 30 min after instillation, small clusters of CF appear in the interstitium (I) subjacent to type II cells. The tracer is located between the basal plasma membrane and the lamina densa of the basement membrane. The amount of CF in this site increases with time as in this 6-hr specimen. ( $\times 56,000$ .) (B) Although closely apposed to type I cells, neither CF nor carbon appears to be internalized by the cells or transported to the interstitial compartment within a 2-hr exposure. ( $\times 65,000$ .) (C) No NF is found in the interstitium adjacent to type II cells within a 2-hr exposure. Type I cells (not illustrated) also fail to transport NF in this time period. ( $\times 65,000$ .)

**Dextran.** Dextran T-70 was observed in small amounts in vesicles, MVBs, and LBs of type II cells with approximately the same time course as CF (Fig. 6 B and C). The Golgi complex was unlabeled, in contrast to this organelle in certain other secretory cells (14, 15). Type I cells did not appear to take up this tracer, and neither cell appeared to transport it to the interstitial compartment, although a positive identification of the tracer in this location may be difficult to achieve.

**Carbon.** Colloidal carbon was observed only in alveolar macrophages. Other cells and the interstitium were unlabeled.

## DISCUSSION

These studies demonstrate that alveolar type II cells are able to internalize, rapidly and in relatively large amounts, certain types of tracer substances and, after internalization, deposit them into MVBs and lamellar bodies. Autoradiographic (16), cytochemical (11), and immunocytochemical (8, 17) evidence indicates that these organelles constitute part of the

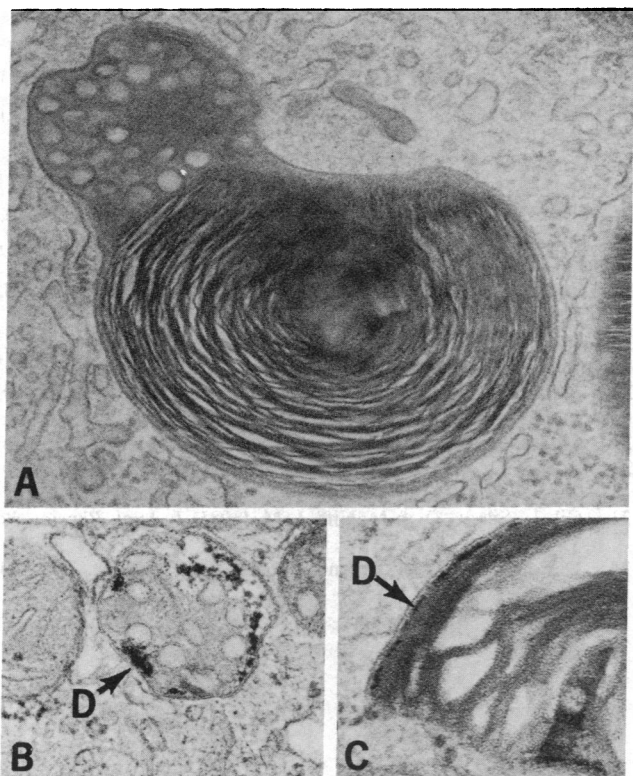


FIG. 6. (A) At 2 hr a few molecules of NF may appear in MVBs of type II cells, but most MVBs and LBs lack tracer as shown here. ( $\times 50,000$ .) (B and C) Dextran (D) is also present in small quantities in a few MVBs and LBs of type II cells after 2 hr. (B,  $\times 48,000$ ; C,  $\times 84,000$ .)

secretory pathway for surfactant, and composite MVB-LB structures are frequently observed in serially sectioned type II cells (18). This study has also tentatively established certain characteristics of the uptake system imposed by the charge and dimension of the molecules used as tracers. Because each animal received identical amounts of protein (CF or NF) and fluid, it was possible to make comparisons of the effective uptake of each tracer by type II cells. As anticipated, cationic molecules (CF) were internalized preferentially and were concentrated intracellularly. Uncharged molecules were taken up in lesser amounts, and anionic molecules (NF) appeared to be largely excluded. This type of discrimination has been observed for several cell types (14, 15) and is most likely due to molecules on the cell surface that carry a net negative charge and permit CF to bind (19). The difference in uptake of NF and CF is important, however, because it indicates that fluid phase pinocytosis contributes less to cellular uptake than do endocytic mechanisms that depend on a prior binding of molecules to the type II cell surface. Like other soluble ligands that bind with high affinity to cell surfaces, CF serves as a membrane marker for as long as it remains tightly bound to internalized membrane. This property has been used by others to follow pathways taken by plasma membrane after endocytosis. In this study, CF was administered as a soluble protein, was rapidly internalized after binding, and then was largely dissociated from membranes as evidenced by its dense packing into electron-dense MVBs. CF uptake therefore may more closely mimic the fate of soluble alveolar constituents than that of type II membranes.

In comparison to CF, NF was taken up by type II cells in negligible amounts. Lauweryns and Baert (12) found large amounts of NF within lamellar bodies of neonatal rabbit type II cells 17 hr after instillation. Although species differences

may account for their findings, a more likely explanation is the marked difference in the amount of tracer used (Lauweryns and Baert, 10 mg/100 g of body weight; this study, 30  $\mu$ g/100 g of body weight). Large quantities of NF may overcome the discrimination between uptake of NF and CF observed in the present study, permitting large amounts of NF to be carried along with any endocytic event.

CF appears to be transported by two different pathways in type II cells. The first of these two pathways transfers material to organelles of the secretory pathway, MVBs and LBs. On the basis of biochemical and morphologic observations, these organelles are often said to be modified lysosomes. MVBs (or a subpopulation) play a special role in type II cells, that of transporting materials from the endoplasmic reticulum and Golgi complex to newly forming lamellar bodies (16). This study establishes as another potential function of MVBs the transportation of materials from alveoli to lamellar bodies after uptake by endocytic vesicles. The presence of large amounts of CF in MVBs within 10–30 min after instillation is suggestive of a very rapid input into the secretory pathway of type II cells. Within 30–120 min, most LBs contain CF, which is presumed to be resecreted when lamellar body contents are released by exocytosis. In the type of *in vivo* experiment done here, where tracers cannot be delivered as a pulse, the tracer molecules are likely to be recycled numerous times until they are eventually cleared by alveolar macrophages. As measured by LB labeling, the minimal time for passage of CF in and out of the cell appears to be about 30 min.

Type II cells do not normally appear to ingest materials by the process of phagocytosis, an endocytic process in which materials of large dimensions [ $0.5 \mu$ m (1)] are internalized, nor do they ingest colloidal carbon when it is administered to normal animals in the small amounts used here. In earlier studies, colloidal carbon was reported to enter lamellar bodies (4), but upon more careful study this was denied (5). Type II cells also failed to internalize polystyrene beads of 1- $\mu$ m diameter (20) or iron oxide particles of 1- $\mu$ m mean length (21). Type II cells normally appear, therefore, to be able to take up tracers with molecular diameters less than 25 nm. These findings, plus the observation that type II cells lack organelles resembling phagocytic vacuoles of macrophages, rule out a phagocytic function.

In addition to being deposited into LBs, CF is transported across the epithelium by type II, but not type I, cells. A similar transepithelial transport of this tracer has been reported for intestinal epithelium (22), another tissue that forms a large interface with the external environment. In lung, this type II cell-mediated pathway has not been previously observed, perhaps because it can be discerned only with a tracer that binds to specific molecules of the apical plasma membrane. Under the conditions used here, this transport appears to be a moderately slow process, with the leading particles appearing in the interstitium after about 30 min. The inability of type I cells to transport any of the tracers tested is perhaps surprising because these cells have been hypothesized to carry out this function in both normal and pathologic circumstances (23, 24), although this is controversial. In comparison to the amount of CF that enters the vesicle-MVB-LB pathway, the second pathway appears to be of minor significance. Whether this transepithelial pathway plays an important role in normal alveolar function is uncertain because most molecules that might be retrieved from alveoli, such as plasma proteins, carry a net negative charge similar to NF.

In summary, these observations indicate that intra-alveolar substances can be transported through type II cells and subsequently deposited into LBs. This pathway, therefore, has the potential to recycle (6) secreted surfactant phospho-

lipid. Whether, in fact, it does so in the normal lung warrants further investigation.

The help of Dr. Leland G. Dobbs, Ms. Lennell Allen, and Ms. Marcia Hansen in carrying out these studies is gratefully acknowledged. Drs. John A. Clements and Susan R. Walker were kind enough to provide helpful criticism of this manuscript before publication. These studies were supported by Program Project Grant HL-24075 from the National Heart, Lung, and Blood Institute.

1. Steinman, R. M., Mellman, I. S., Muller, W. A. & Cohn, Z. A. (1983) *J. Cell Biol.* **96**, 1-27.
2. Brown, M. S., Anderson, R. G. W. & Goldstein, J. L. (1983) *Cell* **32**, 663-667.
3. Pastan, I. H. & Willingham, M. C. (1981) *Science* **214**, 504-509.
4. Niden, A. H. (1966) *Science* **158**, 1323-1324.
5. Corrin, B. (1969) *Thorax* **24**, 110-115.
6. Hallman, M., Epstein, B. L. & Gluck, L. (1981) *J. Clin. Invest.* **68**, 742-751.
7. Williams, M. C. (1982) *J. Cell Biol.* **95**, 388a (abstr.).
8. Williams, M. C. & Benson, B. J. (1981) *J. Histochem. Cytochem.* **29**, 291-305.
9. Williams, M. C. (1977) *J. Cell Biol.* **72**, 260-277.
10. Simionescu, N., Simionescu, M. & Palade, G. E. (1972) *J. Cell Biol.* **53**, 365-392.
11. Goldfischer, S., Kikkawa, Y. & Hoffman, L. (1968) *J. Histochem. Cytochem.* **16**, 102-109.
12. Lauweryns, J. M. & Baert, J. H. (1974) *Ann. N.Y. Acad. Sci.* **221**, 244-275.
13. Brody, J. S., Vaccaro, C. A., Gill, P. J. & Silbert, J. E. (1982) *J. Cell Biol.* **95**, 394-402.
14. Farquhar, M. G. (1978) *J. Cell Biol.* **77**, R35-R42.
15. Herzog, V. & Miller, F. (1979) *Eur. J. Cell Biol.* **19**, 203-215.
16. Chevalier, G. & Collet, A. J. (1973) *Anat. Rec.* **174**, 289-310.
17. Sueishi, K., Tanaka, K. & Oda, T. (1977) *Lab. Invest.* **37**, 136-142.
18. Young, S. L. & Crapo, J. D. (1982) *Am. Rev. Respir. Dis.* **125**, 205 (abstr.).
19. Danon, D., Goldstein, L., Marikovsky, Y. & Skutelsky, E. (1972) *J. Ultrastruct. Res.* **38**, 500-510.
20. Esterly, J. R. & Faulkner, C. S. (1970) *Am. Rev. Respir. Dis.* **101**, 869-876.
21. Sorokin, S. P. & Brain, J. D. (1975) *Anat. Rec.* **181**, 581-626.
22. Rodewald, R. & Abrahamson, D. R. (1982) *Ciba Found. Symp.* **92**, 209-232.
23. Gil, J., Silage, D. & McNiff, J. M. (1981) *J. Appl. Physiol.* **50**, 334-340.
24. DeFouw, D. O. & Berendsen, P. B. (1978) *Circ. Res.* **43**, 72-82.